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A specific binding site for neoxanthin in the monomeric antenna proteins CP26 and CP29 of Photosystem II

Stefano Caffarri[a], Francesca Passarini[b], Roberto Bassi[c], Roberta Croce[b,*]

[a] *Laboratoire de Génétique et Biophysique des Plante, UMR 6191, CEA-CNRS-Université de la Méditerranée, 163 Av. de Luminy, 13288 Marseille, France*

[b] *Department of Biophysical Chemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747AG Groningen, The Netherlands*

[c] *Dipartimento Scientifico e Tecnologico, Università di Verona, Strada Le Grazie 15, 37134 Verona, Italy*

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Abstract The location of the neoxanthin binding site in CP26 and CP29 was investigated by site-directed mutagenesis. The crystallographic structure of LHCII shows that the binding of neoxanthin to the N1 site is stabilised by an H bond with a tyrosine in the lumenal loop. This residue is conserved in CP26 and CP29. Mutation of this tyrosine into phenylalanine induced specific loss of neoxanthin without affecting violaxanthin binding. In contrast to previous proposals, it is thus concluded that also in these minor antenna complexes neoxanthin is accommodated in the N1 site. The characteristics of this binding site in the different antenna complexes are discussed.

Keywords: Photosynthesis; CP29; CP26; Neoxanthin; Light-harvesting complex; Photosystem II

1. Introduction

In higher plants light energy is harvested by the antenna complexes and transferred to the reaction centre where it is used for charge separation. The outer antenna complexes belong to the light harvesting complex (Lhc) multigenic family [1]. They share high sequence similarity and coordinate chlorophyll (Chl) *a*, chlorophyll *b* and carotenoids (Car) in different ratios. Analysis of the crystal structure of LHCII, the main antenna complex of Photosystem II, shows the location of four Car binding sites [2]. Two (L1 and L2) are in the centre of the molecule forming a cross brace between helices B and A, they accommodate lutein (lut) and a small amount of violaxanthin (viola) [3,4]. A third side (N1) located near the C helix is specific for neoxanthin (neo) [5] and in general for *cis* carotenoids [6,7]. A fourth site (V1), located at the periphery of the complex, accommodates violaxanthin and lutein and, in stress conditions, zeaxanthin [8,9]. In this last site, the xanthophyll is only loosely bound, does not transfer excitation energy to the Chls and it is lost upon detergent/acid treatment [8]. Dimeric Lhca complexes of Photosystem I and the monomeric Lhcb protein CP24 cannot bind neoxanthin and have a lower xanthophyll content, hosting two or three xanthophyll molecules depending on the gene product [10]. CP26 and CP29, two monomeric antenna proteins, bind neoxanthin and yet the total number of xanthophylls per molecule is close to 2 [11–13], which led to the suggestion of a mixed affinity of the L2 site for neoxanthin and violaxanthin. However, the Chl/xanthophyll ratio in different preparations of CP26 and CP29 can vary, which suggests that the specificity of the binding sites in these complexes is lower as compared to LHCII [9,12–18] and/or that an additional binding site is present with a lower binding affinity. The distribution of individual xanthophyll species among different binding sites is still controversial: Ruban et al. proposed that the neoxanthin is located in site N1 in both CP26 and CP29 while site L2 site is empty in CP26 [9]. Evidence from in vitro-reconstitution experiments suggests that more than two binding sites might be present in CP26, with neoxanthin being accommodated in both L2 and N1 [12]. In the case of CP29, a chimeric LHCII-CP29 complex in which the C helix domain of LHCII was substituted with that of CP29 was shown to be unable to bind neoxanthin leading to the conclusion that neoxanthin is located in site L2 of CP29 [19].

From the crystal structure of LHCII it could be inferred that the neoxanthin forms an hydrogen bond (H bond) with a tyrosine located in the lumenal loop [2,20], thus stabilising its binding (Fig. 1A). Sequence analysis (Fig. 1B) has revealed that this Y is conserved in all Lhcb proteins but Lhcb6, thus correlating with the presence of neoxanthin. This Y residue is also conserved in Lhca1 in which a small amount of neoxanthin has been observed upon in vitro reconstitution [21,22], although this xanthophyll does not seem to be a pigment component of any Lhca complex in vivo [21].

The correlation between the presence of the lumenal Y and the ability to coordinate neoxanthin suggests the existence of the same specific N1 binding site in all complexes. To check this hypothesis we have performed site-directed mutagenesis

*Corresponding author. Fax: +31 503634800.
E-mail address: R.Croce@rug.nl (R. Croce).

Abbreviations: CD, circular dichroism; Chl, chlorophyll; Car, carotenoid; β-DM, *n*-dodecyl-β-D-maltoside; CP29, CP26, CP24, minor antenna complexes of 29, 26, and 24 kDa, also called Lhcb4, Lhcb5 and Lhcb6, respectively; H bond, hydrogen bond; Lhc, light harvesting complex; LHCII, major light-harvesting complex of Photosystem II, composed by the Lhcb1, 2 and 3 subunits; lut, lutein; neo, neoxanthin; viola, violaxanthin

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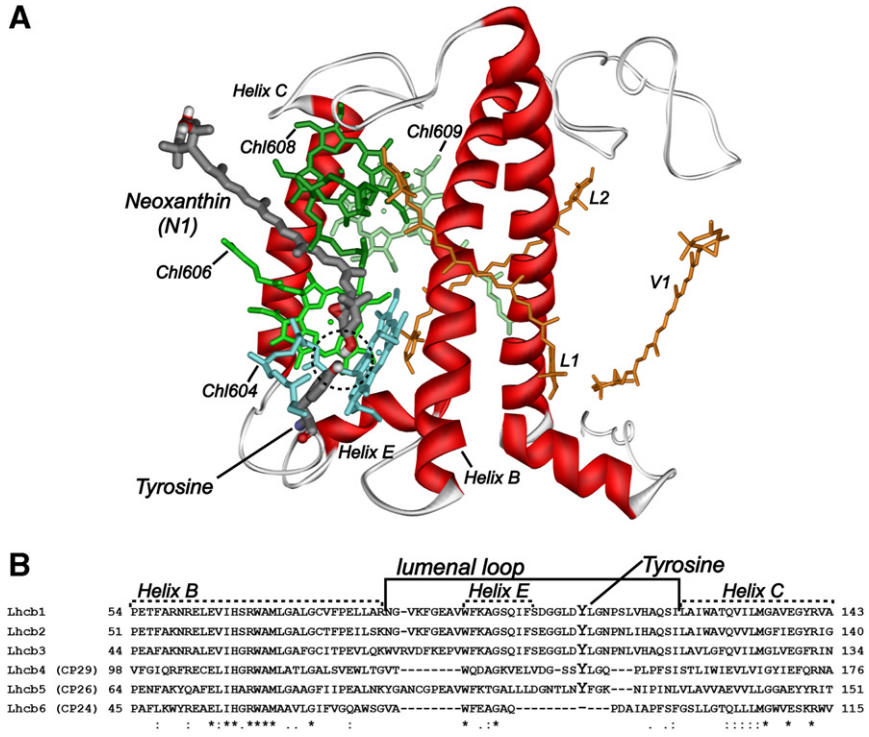

Fig. 1. (A) 3D structure of Lhcb1 showing the H bond between the hydroxyl of the lumenal tyrosine and the C30-hydroxyl of neoxanthin (Tyr112 in the LHCII structure from spinach, [2]). The Chls of this domain and the other Car binding sites are also shown. (B) Sequence alignment of neoxanthin binding domains (Helix B–lumenal loop–Helix C) of Lhcb1, CP26 and CP29. The tyrosine involved in the H bond with neoxanthin is highlighted. The same region of CP24 (Lhcb6), lacking this tyrosine, is also shown.

and substituted the tyrosine with phenylalanine in CP29, CP26 and, as a control, in Lhcb1.

2. Materials and methods

2.1. DNA constructions, recombinant proteins overexpression and pigment–protein complex reconstitution

Lhcb1.3 (AT1G29930), *lhcb4.1* (CP29, AT5G01530) and *lhcb5* (CP26, AT4G10340) mature sequences from *A. thaliana* were amplified from cDNA by PCR and cloned in a modified pET-28a (+) carrying a minimum polylinker. Mutant sequences were obtained by site-directed mutagenesis of the codons for tyrosine 111 (Lhcb1), 147 (CP29), 122 (CP26) to phenylalanine. WT and mutant apoproteins were overexpressed in the Rosetta2 strain of *Escherichia coli* and purified as inclusion bodies. Reconstitution and purification of pigment–protein complexes were performed as described in [3] using a mix of purified pigments with Chls *a*/*b* ratio of 2.9 and Chls/Cars ratio of 2.7.

2.2. Spectroscopy and pigment analysis

Absorption spectra were recorded using a Cary4000 (Varian Inc.) spectrophotometer at a Chl concentration of about 6 μg/ml in 10 mM HEPES, pH 7.5, 0.5 M sucrose and 0.06% *n*-dodecyl-β-D-maltoside (β-DM). Fluorescence excitation spectra were measured at the maximum emission wavelength using a Cary Eclipse (Varian Inc.) spectrofluorimeter and corrected for the instrumental response. Samples were diluted at a Chl concentration of 0.1 μg/ml in 10 mM HEPES, pH 7.5 and 0.03% β-DM. The bandwidths were 2.5 nm in excitation and 10 nm in emission.

The circular dichroism (CD) spectra were measured on a AVIV 62ADS spectropolarimeter. The samples were in the same solution and concentration as described for the absorption.

The pigment complement of the complexes was analyzed by fitting the acetone extract spectrum with the spectra of the individual pigments [12] and by HPLC analysis [23].

3. Results

From the structure of LHCII it could be inferred that the neoxanthin binding in site N1 is stabilised by an H bond with a tyrosine (Y111 in the Arabidopsis Lhcb1 sequence) located in the lumenal loop [2,20]. This Y is conserved in CP26 and CP29, the only other antenna complexes which coordinate neoxanthin. With the aim of verifying the involvement of this residue in neoxanthin binding in CP26 and CP29, the Y was mutated into F in these two proteins and, as a positive control, in the LHCII component Lhcb1. The mutation was designed to abolish the putative H bond between the protein and the neoxanthin, without disturbing the structure of the domain hosting the binding pocket.

The mutant and WT sequences were overexpressed in *E. coli* and the apoproteins were reconstituted in vitro with purified pigments. To ensure an equal availability of pigments for all proteins during the reconstitution, the same pigment mixture was used (see Section 2). In all cases a reconstituted monomeric complex was obtained. The reconstitution yield of the mutants was nearly identical to those of the WTs, thus confirming that the mutation does not influence the stability of the complexes.

The pigment content of the samples is reported in Table 1. The mutation induces loss of neoxanthin in all complexes, albeit to different extents: Lhcb1 loses 16% of the neoxanthin, while this value is 23% and 79% for CP26 and CP29, respectively. The Chl *a*/*b* ratio and the amount of violaxanthin and lutein were not significantly affected by the mutation, demonstrating that the Y vs. F substitution has a specific effect on the neoxanthin binding.

Table 1
Pigment composition of recombinant and mutant (YF) Lhcb1, CP26 and CP29 complexes

Sample	Chl *a*/*b*	Chl/car	Neo	Viola	Lutein	Chl total	Car total
Lhcb1-WT	1.43	4.19	0.86	0.24	1.77	12	2.87
Lhcb1-YF	1.47	4.29	0.72	0.24	1.83	12	2.80
CP26-WT	1.92	3.88	0.85	0.08	1.40	9	2.32
CP26-YF	1.91	4.42	0.65	0.05	1.34	9	2.04
CP29-WT	1.98	3.51	0.70	0.59	0.99	8	2.28
CP29-YF	2.00	4.95	0.15	0.54	0.92	8	1.62

The values are normalized to the total Chl content per monomer as reported in [18]. The error is less than 5%.

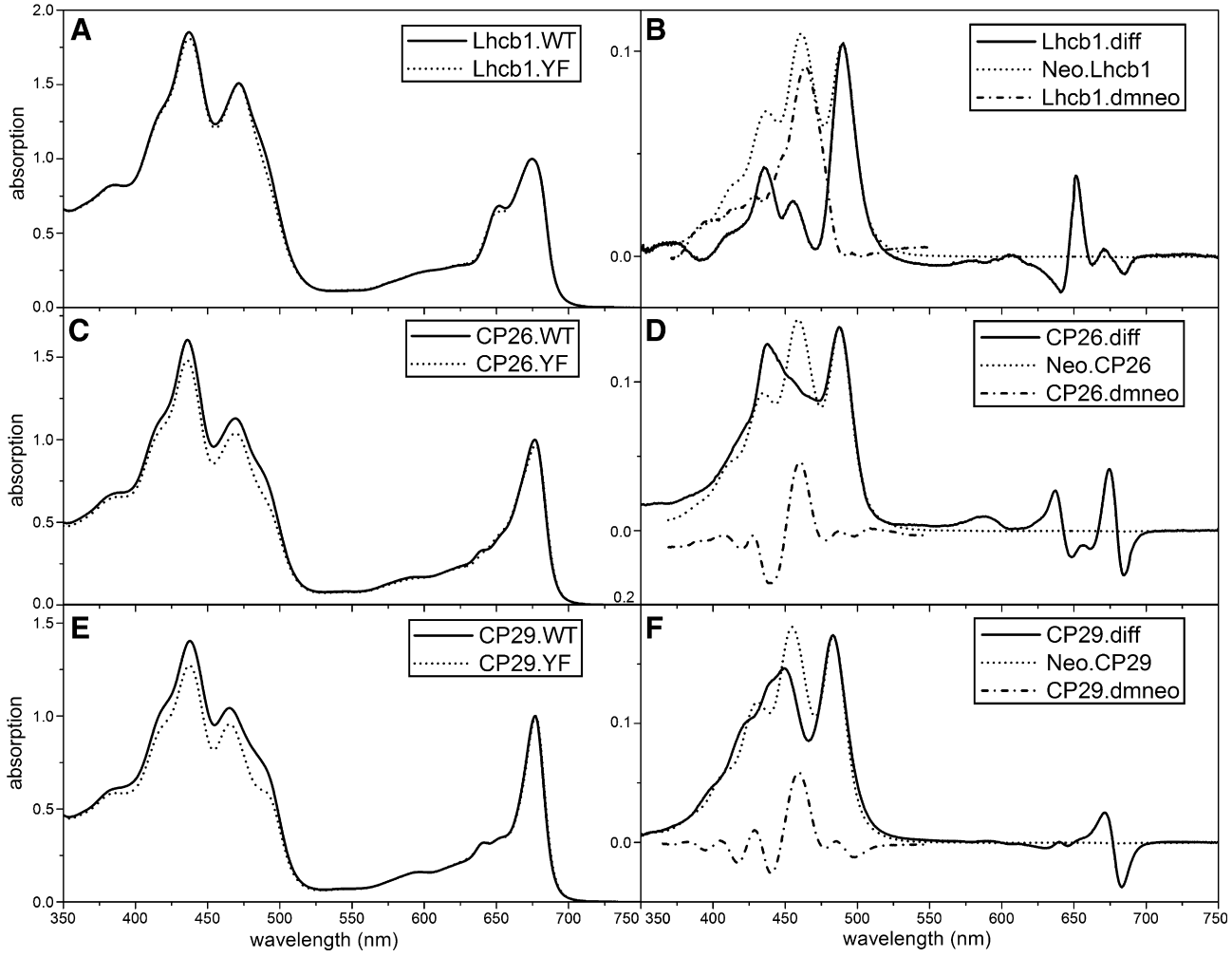

Fig. 2. Panels A, C and E: absorption spectra of recombinant WT and mutant (YF) Lhcb1, CP26 and CP29 complexes, respectively. Panel B, D and F: (WT–YF) difference spectrum (diff, solid); fitted neoxanthin spectrum (Neo, dot); fitted neoxanthin spectrum minus (WT–YF) difference spectrum (dmneo, dash-dot) of Lhcb1, CP26 and CP29, respectively. Spectra were measured at room temperature.

To investigate the spectroscopic characteristics of the neoxanthin in the three complexes, the absorption spectra at room temperature were measured (Fig. 2). Differences can be observed in the blue region, where the carotenoids directly absorb. The amplitude of the differences increases in the order Lhcb1 < CP26 < CP29, in agreement with the pigment determination. Changes were also observed in the Q_y region, indicating that neoxanthin influences the absorption of surrounding Chl molecules (Fig. 2A, C and E). The absorption difference spectra are presented in Fig. 2B, D and F. In Lhcb1 there is a shift in Chl *b* absorption from 652 nm to 640 nm upon mutation. In the blue region the difference spectrum shows a major band at 490 nm with the shape of the red most band of the neoxanthin. However, the two higher energy vibronic bands of the xanthophyll cannot be resolved. To investigate this point, the spectrum of purified neoxanthin was superimposed on the difference spectrum and normalised at 490 nm (Fig. 2B). The match is perfect in the 480–520 nm range, while below 480 nm the intensity of the difference spectrum is far smaller than that of the neoxanthin, suggesting that the mutation created also new absorption in this region. The difference between the expected difference spectrum (e.g. the

spectrum of neoxanthin) and the measured one, has the shape of the Soret band of a Chl *b* with maximum at 463 nm (Fig. 2B). Because the pigment analysis shows that the mutation does not affect the Chl binding, it can be concluded that the loss of neoxanthin changes the spectroscopic properties of a Chl *b* molecule, leading to an increase of its absorption in the Soret region. This effect has previously been observed in Lhcb1 complexes reconstituted in the absence of neoxanthin [5].

Similar results were obtained for CP26 (Fig. 2D). In this complex the absorption maximum of the neoxanthin was found at 488 nm. Increased absorption in the mutant was observed at 460 nm and could be attributed to a Chl *b* as in Lhcb1. In addition, a negative signal was detected at 440 nm, suggesting that a Chl *a* molecule is also influenced by the loss of neoxanthin. Analysis of the Q_y region shows absorption shifts in both the Chl *a* and Chl *b* region thus confirming this suggestion.

In CP29 the absorption maximum of neoxanthin was found at 483 nm, 5–6 nm blue shifted as compared to CP26 and Lhcb1. Also in this complex the Soret band of a Chl *b* at 459 nm becomes more prominent upon mutation. In the Q_y region the spectrum is dominated by a shift of absorption from 670 nm to 682 nm, also indicating a change in the environment of a Chl *a* molecule.

To check the involvement of the neoxanthin in energy transfer, the fluorescence excitation spectra were recorded (Fig. 3). The excitation difference spectra between WTs and mutants were essentially identical to the absorption difference spectra, thus indicating that the neoxanthin is active in energy transfer in all complexes.

To get more insight into the interactions between neoxanthin and neighbouring pigments, CD spectra were measured (Fig. 4). The effect of the partial loss of neoxanthin in Lhcb1 is very similar to what was reported previously for a Lhcb1 complex reconstituted in the absence of this xanthophyll [5]: in the mutant the relative intensity of the bands at 474 and 493 nm is inverted as compared to the WT. In the Q_y region a loss of an excitonic interaction between a Chl *a* (673 nm) and a Chl *b* (652 nm) was also observed. In CP29 and CP26 major changes were detected in the blue region, with the loss of the negative signal at 478 nm in both complexes and an increase of the negative contribution at 497 nm in CP29. In the Q_y region a decrease of the negative signal at 641 nm was observed in both complexes, although more pronounced in CP26.

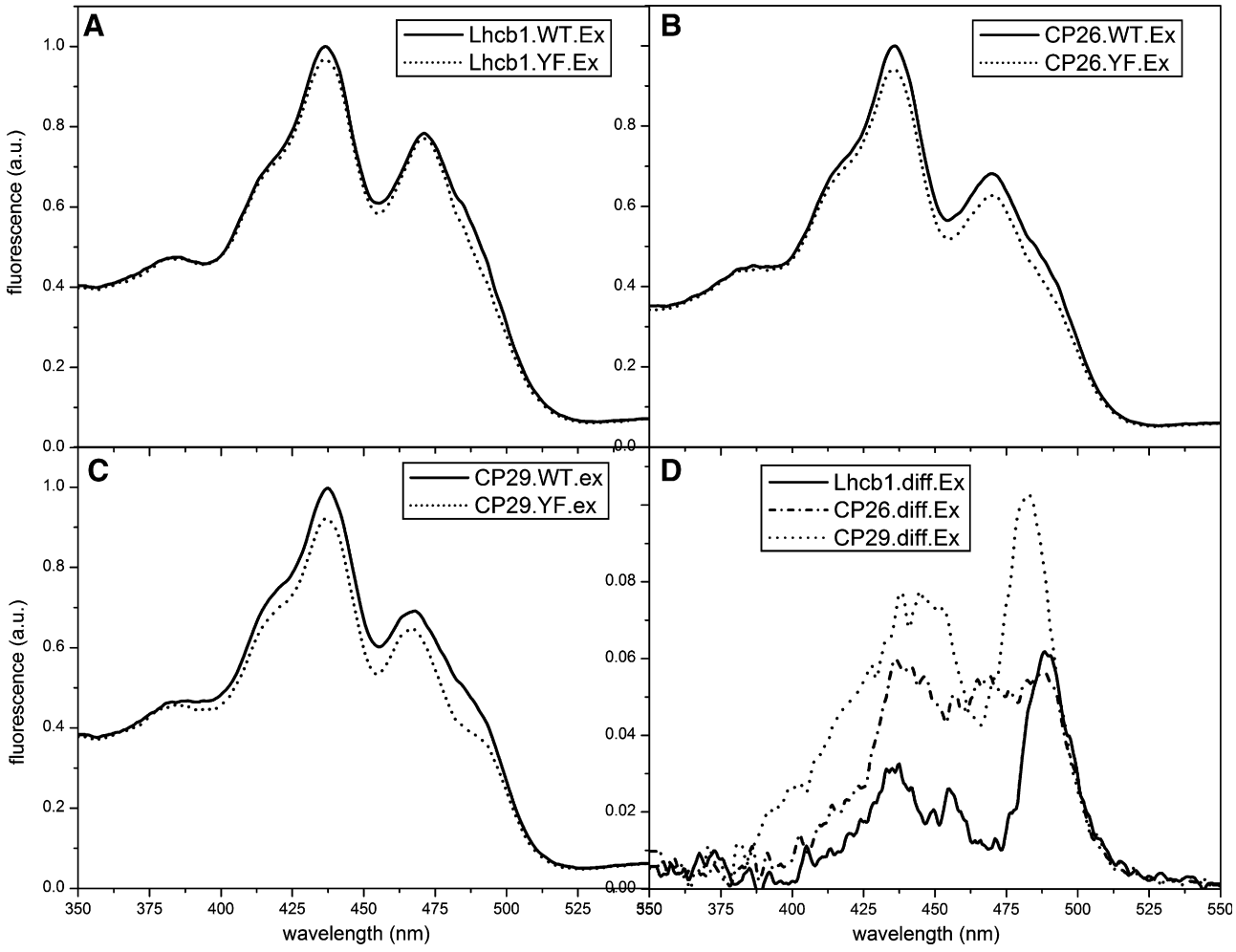

Fig. 3. Panels A–C: excitation spectra of WT and mutant (YF) Lhcb1, CP26 and CP29 complexes, respectively. Panel D: excitation difference spectra (WT–YF) of Lhcb1 (solid), CP26 (dash-dot) and CP29 (dot) complexes.

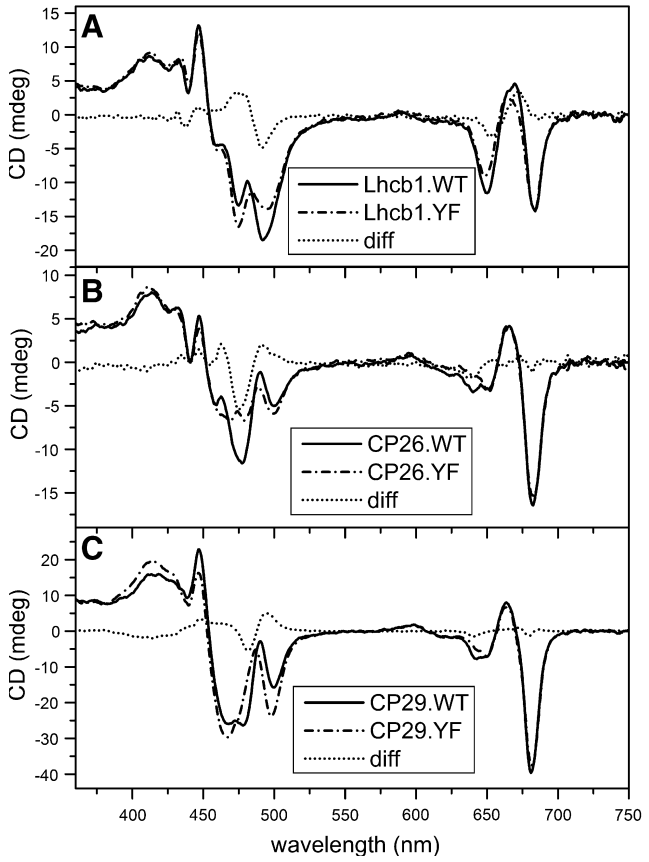

Fig. 4. Panels A–C: circular dichroism spectra of WT and mutant (YF) Lhcb1, CP26 and CP29 complexes, respectively. The difference spectra (WT–YF) are also shown. WT and mutant CD spectra are normalized to the same chlorophyll concentration.

4. Discussion

In all complexes the conservative substitution of Y with F, which eliminates the H bond, leads to preferential loss of neoxanthin. It can be concluded that the N1 binding site is present in both CP29 and CP26, in agreement with a previous suggestion [9]. However, the data suggest that this site differs slightly in these complexes.

4.1. Neoxanthin in Lhcb1

In the absence of the H bond Lhcb1 is still able to retain most of the neoxanthin, indicating that the binding site is also stabilised by other factors. In the difference CD spectrum the loss of an excitonic interaction between a Chl *a*-673 nm and a Chl *b*-650 nm was detected. The best candidates are Chl 604 and 606, both located in close proximity of the N1 site and forming and excitonic pair [24]. Their position is probably slightly different in the mutant. Excitonic calculations showed that these two Chls contribute to the CD spectrum at these wavelengths [25] and in the absence of the interaction an increase of the Soret absorption of Chl *b* 606 is expected (Georgakopoulou and Croce, unpublished), thus explaining the "new" absorption 463 nm in the mutant. In the blue region of the CD spectrum the loss of the conservative signal with contributions at 490 and 477 nm is attributed to loss of an excitonic interaction between the low-energy vibronic band of the neoxanthin (abs 490 nm) and the Soret B_x band of neighbouring Chls *b*, 608 and 606 [25].

4.2. Neoxanthin in Lhcb5

The characteristics of the N1 binding site in CP26 are similar to those of Lhcb1 but not identical. The neoxanthin absorbs at 488 nm and changes in both the Chl *b* and Chl *a* spectroscopic properties are visible. This indicates that the C helix domain in CP26 has a higher Chl *a* content with respect to Lhcb1, in agreement with a higher Chl *a*/*b* ratio.

The Chl *a*–Chl *b* interaction observed in the CD spectrum of Lhcb1 was not detected in CP26, thus suggesting that the Chl 604–Chl 606 pair is not interacting (at least the interaction is not visible in CD), because of a different geometry or because of the absence of Chl 604. This can also explain the difference in the Q_y region between the CD spectra of CP26 and LHCII. The loss of a negative signal at 641 nm in the CD spectrum can be attributed to a Chl *b* interacting with the neoxanthin, possibly Chl 606. The same Chl is probably responsible for the shift from blue to red (637–648 nm) – opposite compared to LHCII – in the absorption spectrum. In the blue region variations in Chl *b* spectroscopic properties are observed, supporting the idea of strong neoxanthin–Chl *b* interactions. Differences in Chl *a* absorption were also detected although the lack of changes in CD suggests that this is not due to a direct interaction with neoxanthin, but rather to a change in the Chl environment.

4.3. Neoxanthin in Lhcb4

The mutation of Y in CP29 leads to the loss of 79% of the neoxanthin. This indicates that the neoxanthin is less strongly bound to CP29 than to the other complexes and its binding is mainly stabilized by the H bond with the lumenal Y. Considering that the C helix domain of CP29 contains less pigments than the other complexes [13,26], the results suggest that pigment–pigment interactions play a role in stabilising the neoxanthin binding. The characteristics of the N1 site in CP29 differ from those of the other complexes, the amplitude of the changes in Chl *a* and Chl *b* properties is rather small, considering that most of the neoxanthin is lost. Moreover, the neoxanthin is blue shifted of 5–6 nm, indicating a different environment and possibly weaker interactions with neighbouring pigments. In the Q_y absorption region the main effect is a shift of Chl *a* absorption towards lower energy, while the Chl *b* region is practically unchanged, confirming that the C helix region of CP29 has a lower Chl *b* content [13]. However, Chl *b*/neoxanthin interactions could be detected in the blue region of the CD and absorption spectra, in a similar way as in CP26, suggesting that the same Chl molecule is involved in interaction with the neoxanthin in both complexes. This is in agreement with time-resolved data which showed excitation energy transfer from a blue Car to a Chl *b* molecule [27], transfer which can now be fully attributed to neoxanthin.

In a previous attempt to localize the neoxanthin binding site in CP29, a chimeric protein was assembled in which the helix C domain of Lhcb1, composed by this helix and the lumenal and stromal loops, was substituted with that of CP29 [19]. This was done considering that the determinants for the neoxanthin binding in N1 should be present in this region. The results clearly showed that the chimeric complex was unable to coordinate neoxanthin, leading to the conclusion that neoxanthin

in CP29 is not bound to site N1. In the present study, thanks to the new information obtained from the higher resolution structure of LHCII, which shows in detail the interactions in the binding site, only a specific residue, Y147, was targeted. The data show that this Y is involved in the neoxanthin binding, not only in LHCII but in CP29 as well. In the chimeric complex the Y was conserved but possibly the match between the two sequences, which have different loops length, was altering the binding site. This is in agreement with the observation that the N1 site in the two complexes differs slightly.

4.4. Lhcb1 vs. CP26 vs. CP29

The analysis of previous purified and refolded complexes gives a relatively broad range of results for the Chl/Car ratio and also for the relative Car ratio in both CP26 and CP29, while the values for Lhcb1 are rather similar. This can be due to the more harsh purification methods used for the minor antenna complexes which leads to loss of pigments, but it is also clearly related to the plant species. Moreover, the occupation of the L1 site is the only one needed to obtain a stable complex, while the other sites can remain empty in the minor antennas [13], thus explaining the observed variability in stoichiometry. This can be necessary during the operation of the xanthophyll cycle to allow the Car exchange in the L2 site and the activation of photoprotective mechanisms [14,28,29]. Moreover, unlike Lhcb1, where in the presence or absence of neoxanthin the sum violaxanthin + lutein is maintained at 2 molecules per complex, in CP29 and CP26 the presence of neoxanthin leads to a decrease in the total amount of violaxanthin + lutein [12,14,19]. This clearly indicates competition between neoxanthin and lutein/violaxanthin in at least one binding site, L2, for the reason discussed above and/or N1, as is the case in Lhcb3 [30]. To give a final answer to these questions a mild purification method, which allows the separation of the individual complexes in the native state, avoiding loss of pigments, is required.

It has recently been shown that the neoxanthin acts as a superoxide anion scavenger at the level of Photosystem II [7]. Its presence in the same site in LHCII, CP29 and CP26 suggests that more than one site is needed at the level of Photosystem II for the scavenging of reactive oxygen species (ROS). This is different as compared to the situation of Photosystem I, which does not contain neoxanthin, and it can be due to the fact that there are no enzymes for ROS scavenging around Photosystem II, which is confined in highly appressed grana partitions, while Photosystem I is easily accessible from the stromal compartment where the enzyme superoxide dismutase is located [31].

The finding that the neoxanthin is present in the N1 site also in CP29 and CP26 can also have important consequences for the interpretation of spectroscopic measurements. In LHCII, it has been shown that a change in neoxanthin configuration correlates with the presence of a quenching state, thus making the neoxanthin an optimum marker for conformational change in vivo [32,33]. It is although possible that similar changes also occur in CP26 and CP29: both this complexes, similarly to LHCII, are able to quench fluorescence upon aggregation [34] and a conformational change in CP26 upon zeaxanthin binding has been shown [28]. We suggest that the question about the localization of the quencher site is still open and thus it would be useful to check the presence of changes in the neoxanthin configuration also in CP26 and CP29.

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